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14. ABSTRACT

RNA viruses can rapidly mutate, causing therapeutics and vaccines to loose their effectiveness. The long-term goal of this project is to predict such mutations, in order to anticipate their effect and design better therapeutics and vaccines. In the funding period reported here, the specific goal was to build a predictive model of viral escape from immune pressure exerted by monospecific T cells in vitro. This goal was achieved: a general model was developed that integrates selective pressure determined by the phenotype of a virus with random mutations at the genotype level. To make quantitative predictions, the model requires parameters characterizing the selective pressure. These were determined experimentally for the specific case

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Report Title

Predicting the Interplay of Epitope Recognition and Evolution in RNA Viruses Under Immune Pressure

ABSTRACT

RNA viruses can rapidly mutate, causing therapeutics and vaccines to loose their effectiveness. The long-term goal of this project is to predict such mutations, in order to anticipate their effect and design better therapeutics and vaccines. In the funding period reported here, the specific goal was to build a predictive model of viral escape from immune pressure exerted by monospecific T cells in vitro. This goal was achieved: a general model was developed that integrates selective pressure determined by the phenotype of a virus with random mutations at the genotype level. To make quantitative predictions, the model requires parameters characterizing the selective pressure. These were determined experimentally for the specific case of T cell recognition of the LCM virus epitope NP 396 – 404. The parameterized model was shown to agree with escape mutants reported to arise when co-culturing the virus with these epitope-specific T cells.

List of papers submitted or published that acknowledge ARO support during this reporting period. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Number of Papers published in peer-reviewed journals: 0.00

(b) Papers published in non-peer-reviewed journals or in conference proceedings (N/A for none)

Number of Papers published in non peer-reviewed journals:

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Number of Presentations:

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

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Number of Manuscripts:

Number of Inventions:

Graduate Students

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Sujan Shresta	0.10	No
Alessandro Sette	0.03	No
FTE Equivalent:	**0.13**	
Total Number:	**2**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Student Metrics
This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: ......

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:······

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:······

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):......

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:......

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense ......

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:......

Names of Personnel receiving masters degrees

NAME
Total Number:

Names of personnel receiving PHDs

NAME
Total Number:

Names of other research staff

NAME	PERCENT_SUPPORTED	
Bianca Mothe	0.17	No
Martin Blythe	0.38	No
Courtney Dow	0.75	No
FTE Equivalent:	**1.30**	
Total Number:	**3**	

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Names of other research staff

NAME	PERCENT_SUPPORTED	
Bianca Mothe	0.17	No
Martin Blythe	0.38	No
Courtney Dow	0.75	No

List of Illustrations and Tables

Statement of the problem studied

RNA viruses can rapidly mutate, causing therapeutics and vaccines to loose their effectiveness. The long-term goal of this project is to predict such mutations, in order to anticipate their effect and design better therapeutics and vaccines. In the funding period reported here, the specific goal was to build a predictive model of viral escape from immune pressure exerted by monospecific T cells *in vitro*. The approach chosen was to build a predictive model based on data from the literature, while simultaneously generating a new experimental data set to parameterize and test the predictive models.

Summary of the most important results

The project goals were specified in five milestones, building on each other.

1) To produce and characterize T cell lines specific for two LCMV epitopes

2) To generate a panel of single substitution analogs of these two epitopes

3) To perform an entropy analysis of the LCMV NP protein by directed sequencing of a large number of viral isolates

4) To construct a model that will predict, based on the data from milestones 2 and 3, which mutations are most likely to appear in wild type LCMV when it is co-cultured with the T cell lines generated in milestone 1

5) To generate *in vitro* LCMV mutants by exerting immune pressure, and verify the accuracy of the prediction generated in milestone 4

The following summarizes the most important results of the project grouped by milestone. For a detailed description including alternative approaches that weren't successful, please refer to the quarterly reports.

Milestone 1) To produce and characterize T cell lines specific for two LCMV epitopes

The milestone was completed successfully. **We established a reproducible source of T cells established**. Of multiple experimental approaches tried, the following protocol gave the best results: Mice where immunized with two known peptide epitopes (NP236 and NP205) from LCMV. The mice were sacrificed 10 days after immunization, and CD8+ T cells were purified from their splenocytes. **The epitope specificity of the T cells was characterized and confirmed by ICCS and ELISPOT assays**. These showed a strong response was induced by NP396, while a low response was induced by NP205. This is in agreement with their previous characterization as a dominant and subdominant epitope **(Figure 1)**.
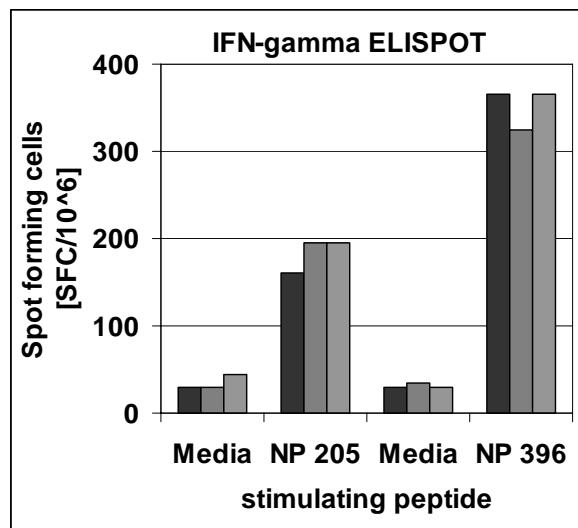
Figure 1: Induction of epitope specific CD8+ T cell response following peptide immunization. Responses were determined in a direct ex vivo ELISPOT assay, and are measured in spot forming cells (SFC) per million CD8+ T cells. The three bars indicate the results from different repeats.

Milestone 2) To generate a panel of single substitution analogs of these two epitopes.

The milestone was completed successfully. **We comprehensively characterized the impact of single residue mutations in epitopes on their T cell recognition.** We synthesized a complete set of single residue substitution peptides, and tested each peptide for the ability to stimulate the T cells generated in milestone 1 in ELISPOT assays (Figure 2). Based on the data, the peptides could be classified into strong, intermediate and weakly

cross-reactive in terms of IFN-gamma production. The pattern clearly shows that that positions 4 – 8 of the NP 396 and positions 1 and 4 of the NP 205 have the strongest influence on T cell recognition.

NP 396 analogs - ELISPOT

	F	Q	P	Q	N	G	Q	F	I
A	0.5	1.0	0.5	0.0	0.5	0.0	0.0	0.0	0.5
C	0.5	1.0	0.0	0.5	1.0	0.0	0.0	0.0	1.0
D	1.0	1.0	0.0	0.5	1.0	0.0	0.0	0.0	0.5
E	0.5	1.0	0.5	1.0	0.0	0.0	0.5	0.0	0.0
F	1.0	1.0	1.0	0.0	0.0	0.0	0.0	1.0	1.0
G	0.0	1.0	0.5	0.0	0.5	1.0	0.0	0.0	1.0
H	0.5	1.0	1.0	0.5	0.0	0.0	0.5	0.0	0.0
I	0.5	1.0	1.0	0.0	0.0	0.0	0.0	0.0	1.0
K	0.0	1.0	0.0	0.5	0.0	0.0	0.0	0.0	0.5
L	0.5	1.0	0.5	0.5	0.0	0.0	0.0	0.0	1.0
M	1.0	1.0	1.0	0.5	0.0	0.0	1.0	0.0	1.0
N	0.5	1.0	0.0	0.0	1.0	0.0	0.5	0.0	0.0
P	0.5	1.0	1.0	0.0	0.0	0.0	0.0	0.0	0.5
Q	0.5	1.0	1.0	1.0	1.0	0.0	1.0	0.0	0.5
R	0.0	0.5	0.0	0.0	0.0	0.0	0.5	0.0	0.5
S	0.5	1.0	0.5	0.0	0.0	0.0	0.5	0.0	1.0
T	0.5	1.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0
V	0.5	1.0	0.5	0.5	0.0	0.0	0.0	0.0	0.0
W	1.0	0.5	0.0	0.0	0.0	0.0	0.0	0.5	0.0
Y	1.0	1.0	0.0	0.0	0.0	0.5	0.0	0.5	0.0

NP 205 analogs - ELISPOT

	Y	T	V	K	Y	P	N	L
A	0.0	1.0	1.0	1.0	1.0	1.0	0.5	1.0
C	0.0	1.0	1.0	0.0	1.0	0.5	0.0	1.0
D	0.0	1.0	0.0		1.0	0.0	0.5	1.0
E	0.0	0.5	1.0	0.0	1.0	0.0	0.5	1.0
F	0.0	0.5	0.5		1.0	0.5	0.5	1.0
G	0.0	1.0	0.0	0.0	1.0	0.5	1.0	1.0
H	0.0	0.5	0.0	0.5	1.0	0.0	0.5	1.0
I	0.0	1.0	1.0	0.0	1.0	0.5	0.5	1.0
K	0.0	0.5	0.0	1.0	0.5	0.0	0.0	1.0
L	0.5	0.5	1.0	0.0	1.0	0.5	0.5	1.0
M	0.0	1.0	1.0	0.0	1.0	0.5	1.0	1.0
N	0.5	1.0	0.0	0.0	1.0	1.0	1.0	0.0
P	0.5	0.5	1.0	0.0	1.0	1.0	1.0	0.5
Q	0.0	0.0	0.5	1.0	0.5	0.5	1.0	1.0
R	0.0	1.0	0.5	0.5	1.0	0.0	0.0	0.5
S	1.0	1.0	0.0	1.0	1.0	1.0	0.5	0.5
T	0.5	1.0	0.0	0.0	1.0	1.0	0.5	1.0
V	0.0	0.0	1.0	0.0	1.0	1.0	0.0	1.0
W	0.5	1.0	0.0	0.5	1.0	1.0	0.5	0.5
Y	1.0	1.0	0.0	1.0	1.0	0.0	0.5	0.0

Figure 2 - T cell cross-reactivity of peptides with single residue mutations from the original epitopes. The top row of each table gives the original epitope sequence. Each cell in the matrix below contains data from a single residue substitution peptide specified by the position of the column in the original peptide and the residues listed in the leftmost column. The color assigned corresponds to strong(green), intermediate (gray) and weakly (red) crossreactive peptides.

Going beyond the originally proposed milestone, we wanted to determine how many of the peptides do not cross-react because they have lost the ability to bind MHC molecules. **MHC binding assays were run for all peptide analogs** to deconvolute the effect of differences in binding affinity with differences in TCR recognition (Figure 3). The positions affecting binding most (such as the C-termini) are not the same as those determining T cell cross-reactivity in Figure 2. This supports the notion of a) limited contacts to TCRs of residues buried in the MHC binding groove, leading the T cells to ignore those substitutions. b) even low affinity binder are able to stimulate a pre-existing T cell response in an IFN-gamma ELISTPOT assay. If the latter would hold in vivo would have to be further analyzed, but is doubtful.

	F	Q	P	Q	N	G	Q	F	I
A	8	4378	12	47	6142	14	6	7576	903
C	104	425	46	36	814	8554	13	450	7362
D	353	752	534	644	1463	39	10	481	14539
E	320	3783	20	63	50000	28	11926	31	50000
F	7	1387	23	417	12572	288	5831	7	792
G	33	9	4	1069	3722	7	8048	561	16074
H	5083	92	3	5637	7986	5	6925	5522	15172
I	4136	804	12	4151	7912	8	4983	99	7
K	4562	679	36	5146	1454	28	302	657	50000
L	228	3907	5558	4921	2410	7735	5631	6489	7531
M	51	9	4395	24	989	9430	5399	398	8646
N	23	124	4591	1314	7	5521	45	158	4462
P	197	490	7	444	9650	6305	4757	826	11025
Q	4246	7	4387	7	4964	6617	7	696	15358
R	9	5693	4799	46	22093	4814	731	926	30427
S	2	4219	20	16	2610	12	3	646	20101
T	7	4428	12	31	25348	5700	6	28	2467
V	34	261	4826	7	21026	33	13	6510	236
W	104	4350	4	93	2615	34	6011	329	27508
Y	26	4472	4924	6097	21234	7	465	4972	33902

NP 396 analogs - IC50 nM

	Y	T	V	K	Y	P	N	L
A	11	27	78	183	1361	9	64	2916
C	366	847	79	564	89	80	576	2269
D	4225	2340	362	189	9740	673	502	11866
E	974	2853	129	38	5818	80	125	50064
F	7	241	0	75	57	13	132	266
G	55	15	51	27	939	46	281	10495
H	159	55	11	20	229	45	100	49344
I	5	90	27	28	3393	8	439	228
K	14	2582	24	60	3226	37	350	48698
L	30	1286	3	12	97	43	64	60
M	12	1318	9	13	114	19	122	36
N	123	1102	56	52	3057	30	60	7577
P	499	3994	55	30	1014	60	271	26397
Q	33	69	7	12	1283	49	152	39872
R	10	3438	13	3	4622	11	31	23132
S	27	12	22	5474	195	18	32	17734
T	42	60	24	55	1980	25	145	19182
V	127	74	60	145	4551	17	373	146
W	68	373	1	16	203	82	549	6980
Y	60	1223	0	83	60	17	39	55140

NP 205 analogs - IC50 nM

Figure 3 - MHC binding affinity of peptides with single residue mutations from the original epitopes. The top row of each table gives the original epitope sequence. Each cell in the matrix below contains data from a single residue substitution peptide specified by the position of the column in the original peptide and the residues listed in the leftmost column. The number in each cell is the competitive binding affinity measured in IC50 [nM]. Weakly binding peptides with IC50 greater than 500nM are colored in red.

Milestone 3) To perform an entropy analysis of the LCMV NP protein by directed sequencing of a large number of viral isolates

This milestone was completed successfully, but much later in the project than originally planned. We were kindly supplied with viral stocks from Jason Botten / Mike Buchmeier at Scripps. These are stocks from a long term culture of LCMV with 60+ passages in MC57 cells. These are many more than passages than we could perform ourselves during this project. We retrieved the initial stock (P0), day 45 (P10) and day 178 (PMax), isolated viral RNA. **Full length genomes were sequenced using standard methods at each time point, which identified the mutations listed in Table 1.**

Protein	Codon	AA change	P10	PMax	(p-illumina)
NP	432	L->F	x	x	**<0.001%**
NP	444	G->D		x	0.11
L	1365	(silent)		x	0.13
L	1711	E->K	x	x	**<0.001%**
L	1713	L->S		x	0.00005

Table 1 - Mutations identified in the LCMV consensus sequence by standard sequencing methods. The last column identifies the p-value associated with these mutations in the Illumina method (Table 2 below).

The number of mutations identified in the consensus sequence even after 63 passages was low, raising concerns that the sequence resolution we were hoping to achieve by sequencing 50+ individual clones would be insufficient. We instead decided to apply novel sequencing techniques that give direct data on the sequence population in a sample without having to isolate clones. This can potentially identify mutations present at a much lower frequency.

We decided to utilize the sequencing services at Illumina, which required less input material compared to the competing 454 technology, and promised a quicker turn around time. Our project was the first applying this technology to viral RNA. Several repeats in the sample preparation and sequencing were necessary before we received results. Those results showed the presence of a Mycoplasma co-infection in the viral culture. As the sequencing technology directly reflects the genomic content in the sample, this led to a reduced amount of LCMV specific sequencing data.

We developed a data cleaning algorithm, selecting sequence reads that a) meet a high illumina quality score cutoff, b) have a significant alignment against the LCMV genome c) have no better alignment against the mycoplasma genome and d) fall within the protein coding region of LCMV. **A total of 1.473k nucleotide calls meet these criteria, of which 98.7% are consensus calls and 1.3% are mutation calls**. If the mutations were randomly distributed sequencing errors, they should follow a Poisson distribution at each nucleotide position. All positions were picked that have a significant higher number of mutation calls than expected by the Poisson distribution with a p-value cutoff $p < 10^{-5}$. This identified 29 nucleotide positions with a high number of mutations present compared to 0.3 that would have been expected by chance (Table 2).

Protein	Codon	mutation	P10	Pmax
GP	33	(silent)	x	
GP	77	V -> A	x	x
GP	203	Y -> H	x	
GP	208	W -> R	x	
GP	424	D -> G	x	
GP	431	S -> N	x	
GP	432	T -> A		x
GP	432	(silent)	x	
NP	160	W -> C	x	
NP	162	V -> G	x	
NP	225	L -> P	x	
NP	300	E -> K	x	
NP	301	N -> D	x	
NP	325	(silent)	x	
NP	325	R -> G	x	
NP	432	L -> F	x	
NP	498	L -> V	x	x
NP	514	I -> T	x	x
NP	515	T -> P		x
L	5	(silent)	x	
L	151	F -> L	x	
L	959	(silent)		x
L	1002	(silent)		x
L	1107	(silent)	x	
L	1711	E -> K	x	
L	2056	(silent)	x	

Table 2 - Mutations identified in the LCMV viral population by the Illumina sequencing method

The sequencing data generated for this milestone corresponds to about 140 full length genomic sequences of LCMV, much more than originally planned. However, due to the change in experimental approach, initial difficulties in the sample preparation and the Mycoplasma co-infection this data was only available at the end of the funding period, and could not be used as planned in milestones 4 and 5.

Milestone 4: To construct a model that will predict, based on the data from milestones 2 and 3, which mutations are most likely to appear in wild type LCMV when it is co-cultured with the T cell lines generated in milestone 1.

$$\dot{n}_i(t) = r(t)\sum_j M_{ij}A_j n_j(t) - \Delta n_i(t)$$

$$r(t) = \eta - r(t)\left(\beta + \sum_j M_{ij}A_j n_j(t)\right)$$

r = resource, η, β = replenishing, depletion rate
n_i = number of viruses i
A_i = Fitness of virus i = *amino acid fitness*
M_{ij} = Mutation j → i = *nucleotide exchange*
Δr = Death rate

Figure 4 - Central rate equation

This milestone was completed successfully. A mathematical model of viral evolution was developed based on combining the theoretical approaches of the Quasispecies and sequence evolution models. The central set of equations is shown in Figure 4.

The primary novel elements of this approach are a) to treat evolution at each site independently b) explicitly model evolution at the nucleotide, codon and amino acid level, c) distinguish between random mutations and fitness mediated selection d) limiting the viral particle counts in steady state to finite numbers by assuming all particles compete for a common resource (such as space). Figure 5 shows a simulation of the total viral particle number dynamics compared to the measured time course.
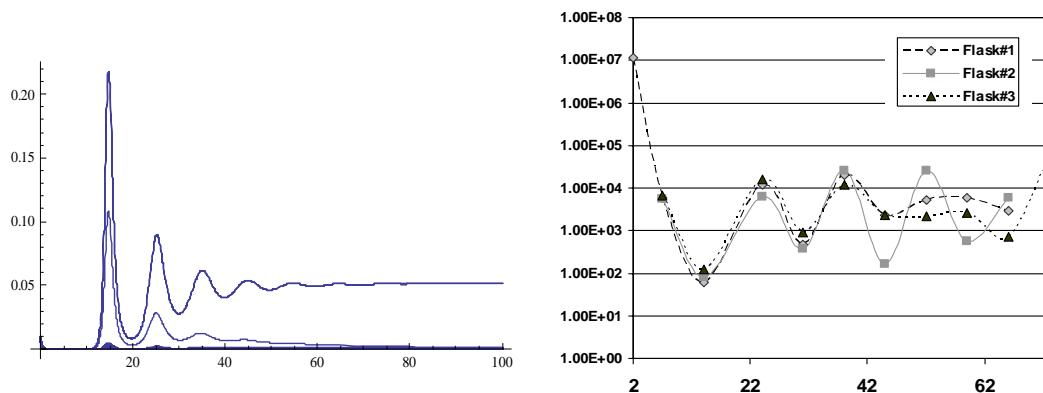

Figure 5 - Dynamics of modeled and measured total viral particle numbers.
The modeled (left panel) and measured (right panel) viral particle numbers (y-axis) are plotted as a function of time t (x-axis). The modeled time courses display relaxation oscillations similar to those observed in the viral titers of the long term LCMV culturing data.

The rate equations given above describe the average dynamic of an infinite number of viral populations. For an individual process, the dynamic expansion has to include stochastic terms, which take into account variations of replication, mutation and destruction events. This was done by introducing Langevin terms into the time-discretized version of the equations. Two separate time courses of simulation runs are shown here:
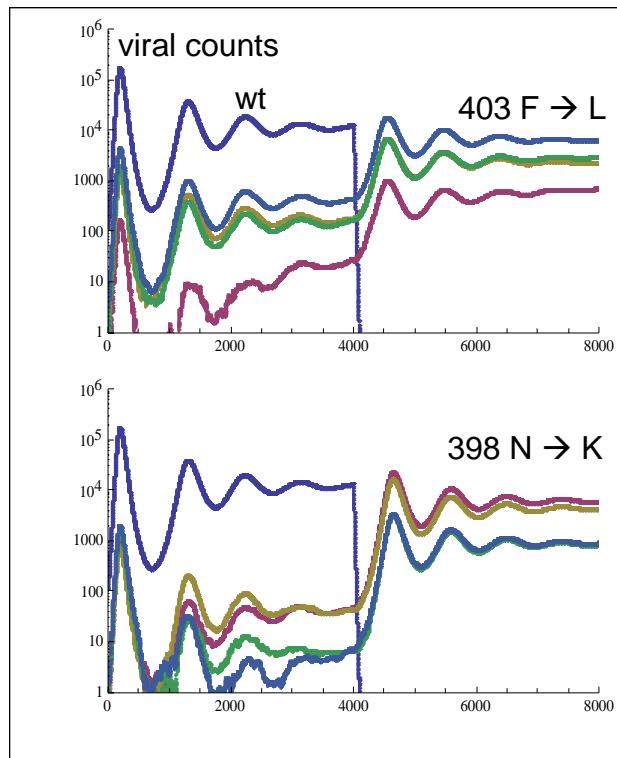

Figure 5 - Dynamics of modeled particle numbers for different members of a viral population. The viral particle numbers (y-axis) are plotted as a function of time t (x-axis). Each colored line represents the viral population associated with a single nucleotide mutation to the wild type (blue). At time 4000, the selective pressure of T cells monospecific for the wild type epitope are added into the model. This gives mutants that are not recognized by T cells an advantage over the wild type sequence.

Repeating these simulations 200 times gives a distribution of escape mutants becoming dominant. Table 3 lists the more frequently observed escape mutations in these 200 repeats. **These are the model predictions utilized in milestone 5 of what escape mutations arise as a result of added selective pressure.**

F	Q	P	Q	N	G	Q	F	I
	R			K	R	L	L	V
	I			A			I	N
	S			V				T
	T							
L	A	H					H	Y
I	S	L					E	
	T							
	L							

Table 3 – Predicted viral escape mutations arising due to immune pressure. The top row gives the wild type epitope sequences. Letters in bold underneath occurred 10 or more times (5%). Letters below at the bottom occurred between 2 and 9 times (1%).

Milestone 5: To generate *in vitro* LCMV mutants by exerting immune pressure, and verify the accuracy of the prediction generated in milestone 4.

This milestone was not completed as planned, as the problems with obtaining sequences from viral cultures were only solved at the end of the project period. As we had resources available, we further optimized the generation of monospecific T cells for the purpose of this milestone. We had purchased MHC tetramers with the NP396 epitope, which allowed us to sort out epitope specific T cells. We compared several experimental approaches to generate epitope specific T cells through an infection first, followed by in vitro restimulation. Optimal results were obtained by infecting mice with recombinant vaccinia viruses expressing the LCMV NP protein, and then expanding the generated T cells through in vitro culture. Figure 5 shows the greatly higher yield of that approach compared to taking cells from LCMV infected animals.
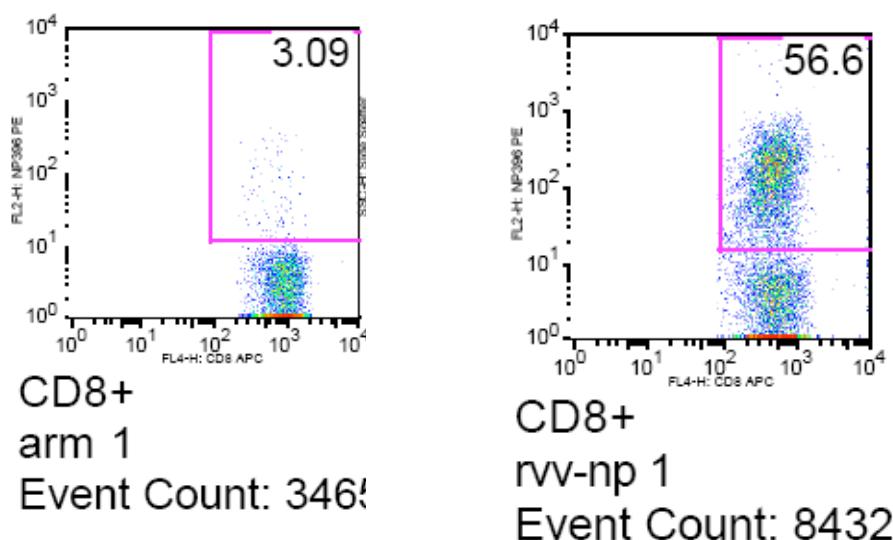

Figure 6 – Optimized protocol to generate monospecific T cells. The panels show FACS tetramer stainings of epitope specific IFN-gamma producing T cells from LCMV infected (left) and recombinant vaccinia virus infected (right) animals.

As an alternative approach to test the predictive capacity of the model from milestone 4, we utilized escape mutation data previously published study by Oldstone [1, 2]. In those experiments, the authors cultured viral populations with T cells monospecific for the NP396 epitope, and isolated viral clones that escaped immune detection. **The mutation identified in the experimental study is NP403 F → L. In our model (table 3), this is also one of the frequently occurring escape mutations. This agreement provides evidence that the mutations predicted in our model are indeed those occurring more frequently in the experiment.**

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Appendixes

N/A